PATENT APPLICATION

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of	Docket No: **Q111431**
Michael GEORGE, et al.	
Appln. No.: **10/551,475**	
Confirmation No.: **4410**	Group Art Unit: **4131**
Filed: **September 30, 2005**	Examiner: **BOESEN, Christian**

For: **FLUORESCENTLY TAGGED LIGANDS**

DECLARATION UNDER 37 C.F.R. § 1.132

Mail Stop RCE
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

We, Stephen Hill and Barrie Kellam declare as follows:

THAT We are a citizen of the United Kingdom;

THAT We are currently employed by University of Nottingham, the original assignee of this application and directors of CellAura Technologies Limited, the current assignee of this application. A copy of our *curriculum vitae* and/or profile is attached hereto.

We further declare and state as follows:

THAT We are two of the named inventors of the invention described and claimed in the above-identified application;

We are familiar with the above-identified application. In relation thereto, we have reviewed the Office action mailed November 27, 2009, in which Claims 47, 64-66, 68, and 91 are rejected under 35 U.S.C. § 103(a) as being unpatentable over McCrea (Molecular

Pharmacology 1996, Vol. 49, pg. 927-937)("McCrea") in combination with U.S. Patent 6,171,794 to Burchard ("Burchard").

We further declare and state that G protein coupled receptors (GPCRs) have been a major focus of drug discovery over the last 40 to 50 years. A historical review of the development of drugs targeted at this large family of receptors is described in Hill, Stephen, *G-Protein-coupled receptors: past, present and future*, British Journal of Pharmacology, 147, S27-37 (2006)(Attached hereto). Since this review, the first crystal structure of a human GPCR, the ß2-adrenergic receptor, has been solved so that accurate modeling of the binding site and pocket is now possible. See also Cherezov *et al.*, *High Resolution Crystal Structure of an Engineered Human ß2-Adrenergic G Protein-Coupled Receptor*, Science, 318(5854), 1258-1265 (2007)(attached hereto).

GPCRs represent a diverse group of receptors, located in the cell membrane, with the ligand binding site for many GPCR receptors located deep within the transmembrane regions of the receptor. What we now know is that the GPCR and a ligand can be equated to a lock and key, i.e., if the key does not fit **and** engage the lock, then the lock is not activated. If a key can fit into the lock and engage the lock, then this key will act as an agonist, and activate the receptor. If however the key fits but does not engage the lock, then this key will act as an antagonist and fail to activate the lock, moreover preventing the binding of the correct key (agonist). What we realized in our work was that if we wished to attach something (a fluorophore) to a key, we would have to do so in a very specific manner to ensure that the key would still fit the lock. As the binding site is located deep within the transmembrane regions of the receptor, this creates a bigger challenge than if the binding site is located more peripherally (as is the case for many peptide GPCRs).

The analysis of GPCRs has largely been confined to studies of radioligand binding or functional activity (cellular responses). In the early years, this was mostly confined to tissue homogenates (for radioligand binding), intact tissues (e.g. strips of isolated smooth muscle) and cultured cell lines. However, although immunohistochemical studies with fluorescent antibodies targeted at epitopes on cell surface proteins have been used to locate GPCRs at the cell surface, it has only been in recent years that fluorescently tagged ligands of GPCRs have been used to identify ligand-bound proteins in cultured cells. This has been partly a consequence of the development of new imaging technologies (e.g. confocal laser scanning microscopy, fluorescence correlation spectroscopy) that allow high resolution and single molecule detection.

Early reports on the synthesis and biological evaluation of fluorescent receptor probes for cell surface receptors were published in the 1970s. For example, in 1974, Anderson *et al.*, *Fluorescent staining of acetylcholine receptors in vertebrate skeletal muscle*. J. Physiol. 237, 385–400 (1974)(attached hereto) reported the labeling, with a fluorescent dye (fluorescein and tetramethylrhodamine), of the snake venom alpha-bungarotoxin, a polypeptide antagonist of molecular weight 8000 Da, that was used for visualization of nicotinic acetylcholine receptors (a ligand-gated ion channel) in vertebrate skeletal muscle fibres. This had the major advantage over immunohistochemical labeling of epitopes on the receptor protein that ligand binding and localization of the receptor could be correlated for the first time. This raised the possibility that the cellular location of a GPCR with a ligand bound to it could also be identified.

This, and many of the early attempts at producing a fluorescent ligand relied on attaching a fluorescent moiety to a large peptide or protein-based ligand which didn't markedly change the overall size or properties of that ligand. However, in the case of small, typically non-peptide,

molecule ligands for GPCRs (which generally have molecular weights in the 500-1000 Da range) this is much more of a major challenge. For radioligand binding studies, the simple substitution of a tritium atom for hydrogen will not change the pharmacological properties of the resulting radioligand. As such, it is fair to assume that the native molecule and the tritiated molecule will behave the same. Unfortunately, in the early years of fluorescent ligand development the same assumption was made for fluorescent small molecule ligands. A number of molecules were made by simply conjugating a fluorophore to a functional group (where available) on the native drug molecule to produce a version that was indeed fluorescent. What was also often not considered was whether their pharmacological properties were altered. For example, Yates *et al. Chemical modification of the naphthoyl 3-position of JWH-015: In search of a fluorescent probe to the cannabinoid CB2 receptor,* Bioorg. Med.Chem. 15, 3758–3762 (2005), describes conjugating the fluorescent dye, nitrobenzodiazole (NBD), directly to the cannabinoid CB2 receptor agonist, JWH-015, but conjugating it with the dye practically abolished the biological activity of the resulting molecule.

The selection of suitable ligand was also often dictated by the presence or absence of a reactive functional group suitable for coupling. Peptide ligands offer readily available functional groups for conjugation, in the form of reactive side chains or the N and C termini. However non-peptide ligands vary in the nature and location of reactive functional groups, if present at all. In some cases these are fortuitously located and can be used for conjugation but in many cases they are either inappropriate or absent. These latter ligands tended to be overlooked as options for fluorescent coupling. For example, we wanted to couple BODIPY 630/650 to salmeterol (as described in Example A3 of the above-identified patent application), and found it necessary to

conduct a *de novo* synthesis in order to conduct the coupling, in view of the lack of suitable coupling sites.

We described one of the first detailed pharmacological characterizations of a fluorescent GPCR ligand (BODIPY-TMR-CGP12177) in Baker *et al, Pharmacology and direct visualization of BODIPY-TMR-CGP: a long-acting fluorescent β2-adrenoceptor agonist*, British Journal of Pharmacology, 139, 232-242 (2003)(attached hereto). We showed that a fluorescent ligand does not have the same biological properties as the native molecule and must be considered as a novel pharmacological agent. This prompted an extensive commentary by McGrath and Daly, *Do fluorescent drugs show you more than you wanted to know?* British Journal of Pharmacology, 139, 187-189 (2003)(attached hereto), which describes the need to properly characterize fluorescent ligands in the manner in which we had done. McGrath and Daly also drew attention to the fact that the slow take-up of fluorescent ligand technologies, despite the arrival of the necessary analytical techniques in the early 90's, could probably be attributed to the fact that many currently available had not been characterized and did not retain their pharmacological activity.

Our characterization was the result of our interest in using fluorescent ligand technology for GPCR visualization. We were, however, convinced that despite the failed efforts in the literature to provide a reliable fluorescent ligand, the ligands could tolerate a bulky fluorophore and the fluorophore could be tuned to give reliable visualization. We also knew that to achieve our aim, we would need to find a strategy that would work for different ligands and different fluorophores. We could see scientific and commercial value in being able to use not just one useful fluorescent ligand, but a fluorescent ligand (agonist, antagonist, inverse agonist etc) for each GPCR.

We therefore wanted to incorporate better spectrally competent fluorophores as GPCR tags. These should incorporate spectrally more appropriate fluorophores (low triplet state, high quantum yields, appropriate band/width). We also appreciated the benefits which could be achieved by visualization of ligand binding using fluorescence correlation spectroscopy, and that the available fluorescent ligands weren't enabling these benefits to be accessed. Finally we wanted to visualize the binding of agonists to non-peptide GPCRs, a greater challenge even than visualizing antagonist binding.

Our design of a fluorescent ligand involves the identification of a suitable native ligand (pharmacophore - agonist or antagonist), a fluorophore (to provide the fluorescence characteristics) and a linker to prevent the fluorophore interfering with the biological activity of the finished product. The combination of pharmacophore, linker and fluorophore to retain biological activity requires careful selection. This is best illustrated by a recent publication by our research group (Baker *et al., Influence of fluorophore and linker composition on the pharmacology of fluorescent adenosine A1 receptor ligands*, British Journal of Pharmacology, 159, 772-785 (2010), detailing the impact of fluorophore and linker on the agonist activity of an adenosine A1-receptor ligand. Here we show that a fluorescent derivative of N-ethylcarboximidoadenosine (NECA) with a four carbon linker, ABEA-X-BODIPY-630/650, retained agonist activity and could be clearly visualized binding to cell surface receptors in living cells. *Id.* Simple substitution of BODIPY-630/650 with Texas Red, EvoBlue or FL-BODIPY resulted in a major loss of pharmacological activity. Clearly, we couldn't simply identify one effective fluorophore and linker combination and apply this to any desired GPCR ligand. Our strategy therefore, was to prepare a mini library usually of about 12 fluorescent ligands, using a set of fluorophores and linkers, for a given GPCR ligand, characterize pharmacologically all of

the library members, and thereby select a fluorescent ligand suitable for conducting binding studies and for visualization. Our set of linkers include, in addition to alkyl of different chain length, oligomeric amide and ether linkers which we can easily manipulate in terms of length, functionality and orientation, and which have good solubility.

In contrast, dansyl derivatives of NECA (with different linker lengths) retained agonist activity, but could not be visualized by confocal microscopy because of heavy quenching of the fluorescence in aqueous environments. The resulting fluorescence observed was no different from the autofluorescence of the cells.

A major advantage of the BODIPY-630/650 fluorophore for adenosine A1 receptors was that it embedded into the membrane environment on binding of ABEA-X-BODIPY-630/650 to the adenosine A1-receptor and became much brighter (Baker et al., supra, 2010). The red nature of this fluorescence also made it highly compatible with visualizing the location of the total receptor pool in parallel using green fluorescent protein (GFP)-tagged receptors. This led us to the benefits of red and blue fluorescent fluorophores which we also illustrated in the figures of our patent application.

Our studies (Baker et al., *supra*, 2010) show that the design of a fluorescent agonist for this receptor requires careful manipulation of the pharmacophore, linker composition and the attached fluorophore in order to generate a molecule with the required pharmacology and fluorescence properties. Simple attachment of a fluorophore to a previously described GPCR ligand is not sufficient and the final constructed molecule requires careful considerations of physicochemical properties, linking strategy and a detailed analysis of the pharmacological properties. This was the basis of our original patent submission: "the design of specifically fluorescent "drugs" with known pharmacological properties. A key to this success is that each

fluorophore has a specific influence on the pharmacology of the resulting product, and it is incorrect to assume that the compound will retain the properties of the non-fluorescent derivative. Important features of the design included the site of binding and the nature of the linking group. The invention therefore provides a method to generate defined and well characterized ligands, having verified properties corresponding to those of the non-fluorescent derivative."

We declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Date: 27-5-2010

S. S. Hill

Stephen Hill

Date: 27-5-2010

Barrie Kellam